

09/914811 PCT

JC05 Rec'd PCT/PTO 3 0 AUG 2001

FORM PTO-1390 (REV. 3-90)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEYS DOCKET NO.	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)				16-007	
INTERNATIONAL APPLICATION NO. PCT/GB00/00761		INTERNATIONAL FILING DATE 03 March 2000 (03.03.00)		PRIORITY DATE CLAIMED 05 March 1999 (05.03.99)	
TITLE OF INVENTION GENETIC SCREENING					
APPLICANT(S) FOR DO/EO/US ARMOUR, John Anthony					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items under 35 U.S.C. 371: 1. ■ This express request to immediately begin national examination procedures (35 U.S.C. 371(f)). 2. ■ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:					
CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
TOTAL CLAIMS		26 - 20 =	6	x \$18.00	= \$108.00
INDEPENDENT CLAIMS		1 - 3 =		x \$80.00	= \$
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				+ \$270.00	\$
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482)\$690.00 <input type="checkbox"/> No International preliminary examination fee paid to USPTO (37 CFR 1.482) but International search fee paid to USPTO (37 CFR 1.445 (a)(2))..... \$710.00 <input type="checkbox"/> Neither International preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$1000.00 <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provision of PCT Article 33(2) to(4)..... \$100.00 <input checked="" type="checkbox"/> International search fee prepared by EPO (37 CFR 1.492(a)(5)).....\$860.00					\$860.00
Surcharge of \$130.00 for furnishing the National fee or oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).					
TOTAL OF ABOVE CALCULATIONS					= \$968.00
■ Reduction by ½ for filing by small entity, if applicable. Applicant is entitled to small entity status under 37 CFR 1.27.					\$484.00
SUBTOTAL					= \$484.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE					\$484.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)) \$40.00					\$
TOTAL FEES ENCLOSED					\$484.00
<p>a. ■ A check in the amount of \$484.00 to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. ■ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0630.</p>					

Express Mail Label No. EL 391522427US

I hereby certify that this paper is being deposited with the
U. S. Postal Service as Express Mail addressed to the
Assistant Commissioner for Patents, Washington, D.C. 20530

8/30/01

Jellen M. Dzulek

3. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
- ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
 - ☒ has been transmitted by the International Bureau.
4. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
5. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
- ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - ☐ have been transmitted by the International Bureau.
6. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
7. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) is enclosed.
8. ☐ A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Other document(s) or information included:

9. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
10. ☐ An assignment document for recording.
Please mail the recorded assignment document to:
- ☐ the person whose signature, name & address appears at the bottom of this page.
 - ☐ the following:
11. The above checked items are being transmitted:
- ☐ before the 18th month publication.
 - ☐ after publication and the Article 20 communication but before 20 months from the priority date.
 - ☐ after 20 months but before 22 months (surcharge and/or processing fee included)
 - ☐ after 22 months (surcharge and/or processing fee included)
- Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 22 months and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
- ☒ by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 - ☐ after 30 months but before 32 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date (surcharge and/or processing fee included.)
 - ☐ after 32 months (surcharge and/or processing fee included).
- Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 32 months and a proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
12. At the time of transmittal, the time limit for amending claims under Article 19
- ☒ has expired
 - ☐ has not yet expired.
13. ☐ Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:
14. ☐ Enclosed is a verified statement claiming small entity status.
15. ☒ Enclosed is an Amendment Before Office Action.

RICHARD A. SHARPE
NAME

WATTS, HOFFMANN, FISHER & HEINKE CO., L.P.A.
P.O. Box 99839
Cleveland, Ohio 44199-0839


SIGNATURE

34,722
REGISTRATION NO.

09/914811

09/914811

JC03 Rec'd PCT/TO 30 AUG 2001

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

ARMOUR, John Anthony

Serial No.: N/A

Art Unit: not yet assigned

Filed: Herewith

Examiner: N/A

For: GENETIC SCREENING

Docket: 16-007

Watts, Hoffmann, Fisher & Heinke Co., L.P.A.
PO Box 99839
Cleveland, Ohio 44199-0839

Box Patent Application
Assistant Commissioner of Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

IN THE CLAIMS

Please cancel claim 1 through 26 without prejudice or disclaimer.

Please add the following claims:

27. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer bonding sites, subjecting the sample to conditions favoring hybridization of the probes to their respective sequences, and amplification of sample-bound probes using a pair of primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample.

Express Mail Number: EL391522427 US
I hereby certify that this paper is being deposited with the
U. S. Postal Service as Express Mail addressed to the
Assistant Commissioner for Patents, Washington, D.C. 20231

8/30/01

John M. Byrd

28. A method according to claim 27, characterized in that each probe is distinguished from the other(s).

29. A method according to claim 27, characterized in that each probe is distinguished from the other(s) by having distinguishing mobility characteristics through a separating gel.

30. A method according to claim 27, characterized in that the plurality of different probes comprises a predetermined set of different probes each chosen to be specific for a respective target nucleic acid sequence.

31. A method according to claim 30, characterized in that the set comprises probes suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced enables quantitative determination of the copy number of the respective sequences in the sample.

32. A method according to claim 27, characterized in that the polymerase chain reaction is used to determine the quantity of each probe product produced.

33. A method according to claim 27, characterized in that the method is used to screen sequences of different genes or different sequences within a gene.

34. A method according to claim 27, characterized in that the method is used to screen sequences of different exons in a eukaryotic gene.

35. A method according to claim 27, characterized in that the method is used to detect genetic alterations.

36. A method according to claim 27, characterized in that the method is used to detect genetic deletions (reductions in sequence copy number) or genetic amplification (increases in sequence copy number).

37. A method according to claim 27, characterized in that the genetic material is immobilized prior to hybridization, such that hybridization flanking primers are likewise immobilized.

38. A method according to claim 27, characterized in that an excess of probes is used.

39. A method according to claim 27, characterized in that probes labelled for ready identification are used.

40. A method according to claim 27, characterized in that probes labelled with fluorescent labels are used.

41. A method according to claim 30, characterized in that more than one set of probes is used, either simultaneously or sequentially.

42. A method according to claim 30, characterized in that more than one set of probes is used and flanking primer pairs are the same for each set of probes.

43. A method according to claim 30, characterized in that more than one set of probes is used and flanking primer pairs are different for each set of probes.

44. A method according to claim 27, characterized in that the method comprises means to obviate or mitigate hybridization between primer binding sequences.

45. A method according to claim 27, characterized in that competing oligonucleotides are introduced to the sample.

46. A method according to claim 27, characterized in that competing oligonucleotides are introduced to the sample during the hybridization stage to releasably bind to primer binding sites flanking each probe to mitigate primer binding site interactions.

47. A method according to claim 27, characterized in that unbound probes and primers are thoroughly washed away from bound probes following hybridization and prior to analysis.

48. A method according to claim 27, characterized in that the method is used to screen one or more of DNA, RNA and cDNA with appropriate probe sets.

49. A method according to claim 27, characterized in that the method is used to screen one or more of somatic and germline sequences.

50. A method according to claim 27, characterized in that the method is used to screen for polymorphic alterations.

51. A set of probes adapted for use in performing the method according to claim 27.


52. A kit for performing the method according to claim 27 comprising a probe set, amplification primers and means to enable amplification and analysis of amplification product(s).

REMARKS

The claims have been amended to eliminate numerous multiple dependencies and to generally put them in a form conducive to prosecution in the United States. Favorable consideration of this application is respectfully requested.

Dated: August 30, 2001

Respectfully submitted,


Richard A. Sharpe
Reg. No. 34,722

Telephone: (216) 241-6700
Facsimile: (216) 241-8151

09444660

[illegible]

The loss or reduction in the normal number of copies of a genetic sequence (deletion) or the increase in copy number (amplification) are of widespread general importance. Such genetic alterations are known to underlie phenotype characteristics both somatic and germline including disorders as diverse as idiopathic mental retardation and neoplasia. The demonstration of the site and nature of such genetic alteration is critical in the identification of the genes responsible and to the development of appropriate and effective treatments and therapies.

In principle, it is possible to screen for genetic deletions by using Southern blot hybridisation. However, this method requires heterozygosity, and whilst the loss of heterozygosity at polymorphic loci can be used to demonstrate the absence of one allele in the soma or in the germline, its effectiveness is limited by the requirement for heterozygosity and the number of repeated tests needed to screen even a small fraction of the genome.

If sufficient precautions are taken to assure quantitative yields, the polymerase chain reaction (PCR) can be used to screen for copy number, and specialised systems have been developed to assure quantitative PCR by following the accumulation of products as amplification proceeds. However, such systems do not provide for the satisfactory analysis of a plurality of alterations substantially simultaneously.

According to the present invention there is provided a method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target

sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample to conditions favouring hybridisation of the probes to their respective sequences, and amplification of sample-bound probes using a pair of primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample.

Preferably each probe is distinguishable from the other(s), for example by having distinguishing mobility characteristics through a separating gel. Preferably the plurality of different probes comprises a predetermined set of different probes each chosen to be specific for a respective target nucleic acid sequence. The set may comprise probes suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced, preferably using the polymerase chain reaction (PCR), enables quantitative determination of the copy number of the respective sequences in the sample. The method may be used to screen sequences of different genes or different sequences within a gene, such as different exons in a eukaryotic gene. Preferably the method is used to detect genetic alterations such as genetic deletions (reduction in sequence copy number) and genetic amplification (increase in sequence copy number).

Preferably the genetic material is immobilised prior to hybridisation, such that hybridised flanking primers are likewise immobilised. Preferably an excess of probes is used.

Probes labelled for ready identification, such as with fluorescent labels are preferably used. More than one set of probes may be used, either simultaneously or sequentially. The flanking primer pairs may be the same or different for each set of probes.

Preferably the method comprises means to obviate or mitigate hybridisation between primer binding sequences. Competing oligonucleotides

may be introduced to the sample preferably during the hybridisation stage to releasably bind to the primer binding sites flanking each probe whereby to mitigate primer binding site interactions.

Preferably unbound probes and primers are thoroughly washed away from the bound probes following hybridisation stage and prior to analysis.

The method may be used to screen DNA, RNA and/or cDNA with appropriate probe sets. The method may be used to screen somatic and/or germline sequences. The method may be used to screen for polymorphic alterations.

The invention further provides a set of probes substantially as described above in any of the preceding seven paragraphs.

The invention may still further comprise a kit for such a method described above in any of the preceding eight paragraphs, which kit comprises a probe set generally as defined above, amplification primers and means to enable amplification and analysis of amplification product(s).

The present invention will now be described by way of example only with reference to the accompanying drawings, in which:-

Fig. 1 is a simple schematic illustration of a method according to the invention;

Fig. 2 shows a gel run produced from samples from four unrelated humans using the method according to the present invention;

Fig. 3 shows an enlargement of the central region III of the gel in Fig. 2;

Fig. 4 shows the results of quantitative analysis of the region III of the gel from one male sample (upper trace) and one female (lower trace);

Fig. 5 shows the relative signal from different probes shown plotted against probe size;

Fig. 6 shows the *EcoRV* cloning site of pZero2 and the corresponding

primer combination (PZA and PZB) used to generate probes from fragments cloned into the *EcoRV* site.

With reference to Fig. 1, there is provided a method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample to conditions favouring hybridisation of the probes to the respective sequences, and amplification of sample-bound probes using a pair of flanking primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acids sequences in the sample.

The method can be used to screen for genetic alterations, in particular deletions and amplifications in copy number of genetic sequences, both somatic and germline.

The sample of genetic material to be screened, which may comprise, DNA, cDNA and/or RNA, shown diagrammatically in Fig. 1 (10), is reversibly immobilised on a medium (12) such as a nylon filter.

A plurality of probes (14) is designed and prepared to seek out and hybridise with specific nucleic acid sequences, if present in the sample. The probes (14) are chosen to comprise a set of different probes each type operable to seek out a different respective nucleic acid sequence which may be sequences suspected of being involved in a particular disorder or condition for example, in the organism from which the sample has been taken. Alterations in the copy number of particular genetic sequences are known to be involved in conditions such as cancer and mental retardation, and probes specific for these sequences can be prepared and comprised in a probe set used in accordance with the present methodology for example where certain symptoms suggest these disorders or diseases may be involved.

When an appropriate probe set is created or identified, it is then introduced to the immobilised genetic material in stoichiometric excess under conditions favouring hybridisation of the probes to their respective nucleic acid sequences, if present.

Once sufficient time has elapsed to provide for hybridisation, unbound probes are washed thoroughly away and the PCR employed to amplify bound probes.

The use of the same primer pair binding sites flanking all the different probes is important to the successful operation of this method, and allows different bound probes to be amplified together with a single primer pair and thus the screening for a plurality of different loci at the same time.

Some interaction has been experienced between primer binding sites flanking different probes, but this problem has been addressed by adding to the hybridisation solution an excess of competing oligonucleotides which releasably bind to the respective primer binding sites thereby preventing such undesirable interactions whilst not adversely affecting the subsequent amplification process. The oligonucleotides are displaced once hybridisation of the probes to the sample is complete, to provide for hybridisation of the amplification primers to the binding sites.

Respective sets of probes can be made either by ligation to oligonucleotide adapters, or more simply by preparing the probes as inserts into the same site of a cloning vector, and using primers flanking the cloning site for amplification. Fig. 6 shows the cloning site of the vector pZero2 (sequence provided by InVitrogen: <http://www.invitrogen.com/vecseq-gcg/pzero2.seq>), showing the primers PZA and PZB flanking the *EcoRV* site (lowercase) used to clone blunt-ended fragments. Products amplified using these primers are therefore 59bp longer than the cloned inserts. It is thus possible to prepare sets of probes, each detecting a different locus, but all flanked by the same primer-binding sites.

It is important that a probe set is created such that each contributing probe is distinguishable, for example by virtue of its mobility through a separating gel. Following amplification, the appearance of a product of corresponding mobility acts to report the presence of a particular nucleotide sequence, and hence the presence of that sequence in the target sample. As an excess of probe is used, and amplification is restricted to the quantitative phase, the absolute amount of each product reflects its copy-number in the sample tested. Different probes may amplify with different efficiencies, but the proportional contribution from each probe will reflect its dosage in the sample and can be inferred from comparisons between different samples examined with the same probe set. As it is the retained probe that is amplified, not the sample, the gel mobility of the product is not influenced by the presence (for example) of Restriction Fragment Length Polymorphism (RFLPs) at that locus.

The following outlines experiments conducted using the method of the present invention to screen a plurality of different genetic sequences simultaneously. A set of 43 amplifiable probes was generated by cloning blunt-ended fragments into the *EcoRV* site of pZero2 (Fig. 6), followed by amplification with vector primers flanking this site, and selection of a mix of probes of different lengths. This mix included probes from 7 autosomes (1, 5, 11, 16, 17, 18 and 22), as well as the X and Y chromosomes.

The initial mix of 43 probes was subcloned from plasmids and plasmid fragments known from sequencing or hybridisation not to contain dispersed repeat elements; in the case of one of the Y-linked probes (SRY), a 1360bp fragment was amplified using primers SRYA (5'GCAGTAGAGCAGTCAGGGAG3') and SRYB (5'GGGGAGAGAAAGAAACAAGTTTG3'). Other sources of cloned genomic DNA were: chromosome 1, pJBT2 (JALA, unpublished); chromosome 5, pMS621 (Armour et al., 1996); chromosome 11, pEJ6.6 (Goldfarb et al., 1982); chromosome 16, pRN6N6A (JALA and R.M. Badge, unpublished); chromosome 17, pYNZ22 (Nakamura et al., 1988); chromosome 18, pMS440 (Armour et al., 1990); chromosome 22, pMS632c (Armour et al., 1995); chromosome X, pMS613 (Armour et al., 1990). After isolation of the genomic insert and digestion with

frequently-cutting restriction enzymes producing blunt ends (generally double digestion with *AluI* plus *HaeIII*), the resulting smaller fragments were cloned into the *EcoRV* site of pZero2 (InVitrogen) and propagated in *E.coli* TOP10 (InVitrogen). Care was taken to avoid repeat regions in DNA from minisatellite-containing clones.

With reference to Fig. 6, probes prepared by cloning blunt-ended restriction fragments from primary clones into the *EcoRV* site of pZero2 (InVitrogen) were amplified directly from bacterial cells (Sandhu et al., 1989) using flanking vector primers PZA (5'AGTAACGGCCGCCAGTGTGCTG3') and PZB (5'CGAGCGGCCGCCAGTGTGATG3'). The positions of these primers in the pZero-2 cloning site is shown in figure 6. PCR was carried out in Advanced Biotechnologies Buffer IV (75mM Tris-HCl pH8.8, 20mM (NH₄)₂SO₄, 0.01% Tween), with 0.2mM each dNTP, 1mM MgCl₂, 0.2μM each primer and 0.05U/μl Taq DNA polymerase (Advanced Biotechnologies). Reactions (generally 10μl) were subjected to 25 cycles of (95°C for 1 minute/70°C for 1 minute). Products amplified using ³³P end-labelled PZA were separated on denaturing 6% polyacrylamide/50% urea gels and probe mixes assembled such that the mobilities of all constituent probes were distinct.

Sample DNA for immobilisation was prepared in an initial volume of less than 5μl, denatured by addition of 1μl 1M NaOH, and spotted onto a nylon filter (MSI MAGNA, approximate dimensions 2mm x 4mm), 1μl at a time, allowing the solution to dry between applications. When all the DNA had been added the DNA was irreversibly bound to the filter using U.V. irradiation. Since numerous filters were to be used in the same hybridisation, individual marks cut into the edges and corners made clear the identity of each filter.

Filters were prehybridised in 1ml of prehybridisation solution (0.5M sodium phosphate pH7.2, 7% SDS, 0.1mg/ml alkali-denatured herring sperm DNA) at 65°C overnight, and before hybridisation this was exchanged for 200μl of prehybridisation solution with the addition of denatured human Cot-1 DNA (Gibco BRL) to a final concentration of 10μg/ml. Probe mixtures containing

about 100pg of each sequence in 1µl were mixed with 7µg *E.coli* DNA (DH5α DNA, digested with *Hae*III) and 1µg human Cot-1 DNA (Gibco-BRL), and denatured by adding 2µl 1M NaOH. After 1 minute at 37°C, the probe mixture was placed on ice, neutralized by adding 3µl 1M NaH₂PO₄, and added to the hybridisation solution.

Hybridisation was left to proceed at 65°C overnight, and the filters were thoroughly washed at 65°C in (a) two 1ml changes of prehybridisation solution, (b) 200ml 1x SSC/1% SDS and (c) 500ml 0.1x SSC 0.1% SDS. Washed filters were then transferred to individual 50µl amplification reactions (constituents as for probe amplification), and bound DNA amplified for 5 cycles of 95°C 1 minute/70°C 1 minute.

This low-level preamplified solution was then used to seed further 10µl amplifications using ³³P 5' end-labelled primer PZA. Labelled PCR products were given a final incubation at 72°C for 20 minutes (to drive to completion the addition of untemplated A at the 3' ends), an equal volume of formamide dye mix (98% formamide, 10mM EDTA pH 8.0, 1mg/ml xylene cyanol FF, 1mg/ml bromophenol blue) added, and the DNA denatured at 100°C for 2 minutes and then placed on ice. From this denatured sample, 4µl was loaded into a well of a 6% polyacrylamide/50% urea gel in 0.5x TBE, and the gel run at 90W (to maintain a gel temperature of about 45°C) until the xylene cyanol dye was close to the end of the gel. Gels were fixed in 10% methanol/10% acetic acid, dried, and radioactivity detected either by standard autoradiography or (for quantitative analysis) using ImageQuant software analysis on data captured by a storage phosphorimager screen (Molecular Dynamics).

The screening results of hybridising this probe set with DNA from one male and three females is shown in figure 2. The autosomal loci give comparable signals, between all individuals; while there is probe-to-probe variation in absolute intensity, the relative contribution of each band to the whole is highly reproducible. By contrast, the Y-derived probes only give strong signals in the hybridisation with male DNA. The relative signal from the X-

linked probes is reduced by about 50% in the male (figures 3 and 4).

In figure 5, the normalised relative signal from each probe is shown plotted against the length of the probe; signal intensities are normalised relative to the mean value for that probe among all individuals tested for autosomal loci, among all the females for X-linked probes and relative to the male for Y-linked probes. The boxed points are the points for X-linked loci in the male (about 50% signal intensity) and for Y-linked probes in females (close to zero). This experiment models the expected detection of homozygous deletions (Y-linked loci in females) or hemizygous deletions (X-linked loci in males). In the size range 140-600bp the signal appears to reflect copy number variation consistently.

Nearly all the amplified bands seen after hybridisation corresponded in size with the expected probes. There were however three bands (marked with asterisks on figure 2) which did not correspond to probes of known origin; the largest of these is clearly Y-linked. The simplest explanation for the appearance of these bands is that they are the result of contamination of the probe mix by other probes made at the same time. Since (if hybridisation has gone to near completion) the intensity of the band recovered from a target DNA will depend on its copy-number in the target DNA, and not on its concentration in the probe mix, even relatively minor contamination with additional probes could result in the appearance of unexpected bands in the amplified product at intensities equal to the predicted bands. Probe fragments corresponding to two of these additional bands are detectable as components of the initial probe mix. The importance of avoiding even minor contamination with additional probes represents an advantage of isolating and storing the probes as clones (rather than PCR products), since uncontaminated probe preparations can be reconstituted from colony-purified clones.

The efficiency of this method derives from its ability to examine numerous loci simultaneously (in this experiment, 43 probes). In the format used here, in which radiolabelled probes are resolved using denaturing

polyacrylamide gel electrophoresis, the number of probes which can be analysed simultaneously is limited only by the resolving power of the gel. Since probes in the size range 140-600bp have been used successfully in this experiment, careful size-selection of probes could lead to probe sets containing more than a hundred probes, which might (for example) be used as chromosome-specific subsets to analyse copy number at megabase resolution.

Moreover, greater resolution may be achieved using fluorescently-labelled probes and apparatus and software designed for automated sequence analysis. This would not only have the advantage of single-base resolution over a larger range (and hence the possibility of more probes per lane), but could also use multiple fluors in a single track. Thus if (for example) four sets of each of 100 probes were assembled, the probes of each set flanked by different primer binding sites (same within a set), they could be hybridised with the same target, and after the initial recovery of bound probes and low-level preamplification using all primer pairs, each set could be selectively amplified with its own primer pair (one fluorescent), and the four resulting sets of fluorescent products mixed and run in the same lane. Similarly, if the same primer was available with different fluorescent labels, then a direct comparison between two samples (for example tumour versus normal from the same person) could be made by labelling the products from each with different fluors and running in the same lane of the gel.

In the present format, the high resolution of polyacrylamide gel electrophoresis allows the simultaneous differentiation of products from many different loci, but even using all the available "slots" with judicious assembly of probe sets, this format probably limits the number of probes flanked by a given primer pair which can be resolved in one lane to about 500, and hence if four probe sets with different flanking primers can be used simultaneously, fewer than 2000 probes can be examined simultaneously in this test. It might be possible to augment the resolution of this analysis by using formats for the quantitative detection of amplified probe which do not rely on gel electrophoresis. One alternative would be to use recovered probes to label

arrays of the clones from which the probes themselves were produced; a procedure analogous to array-CGH (Pinkel et al., 1998), but with the added efficiency that the method according to the present invention the MAPH has already produced proportionate amounts of the relevant single-copy probes from the genomic targets, reducing the complexity of the mixture (for increased speed of hybridisation) and by avoiding multi-copy sequences, presumably allowing quantification with a greater signal:noise ratio.

The experiments explained here have used genomic DNA as the target nucleic acid, but an extension of this method would be to use probes to probe RNA (or cDNA) for highly parallel analysis of gene expression. Current RT-PCR based methods for quantification of gene expression do not allow the simple analysis of numerous loci simultaneously.

The method could be used to screen for alterations to a plurality of sequences relating to different genes, and/or sequences within a target gene e.g. screen for alterations in exon copy number within a eukaryotic gene.

Using the method according to the present invention to detect deletions and amplifications in (for example) idiopathic mental retardation (IMR) assumes that the absence of a locus is abnormal, rather than resulting from a polymorphism. This can be most simply investigated by analysis of the parents in cases of IMR, but it remains possible that wider application of this technique will uncover loci at which there are presence/absence polymorphisms. If enough (sufficiently informative) loci of this kind were available, then assembly of such probes into a mutually compatible "polymorphic set" would allow simultaneous genotyping at (say) 50-100 loci in a single lane of a sequencing gel; combined with analysis of mixed DNA samples, such a system could provide very high throughput for genotyping without the need for investment in expensive or complicated equipment.

Whilst endeavouring in the foregoing specification to draw attention to those features of the invention believed to be of particular importance it should

be understood that the Applicant claims protection in respect of any patentable feature or combination of features hereinbefore referred to and/or shown in the drawings whether or not particular emphasis has been placed thereon.

CLAIMS

1. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material (10), the method comprising introducing to the sample (10) a plurality of different genetic probes (14) suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample (10) to conditions favouring hybridisation of the probes (14) to their respective sequences, and amplification of sample-bound probes (14) using a pair of primers, wherein analysis of the respective amounts of amplified probe (14) provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample (10).
2. A method according to claim 1, characterised in that each probe (14) is distinguishable from the other(s), for example by having distinguishing mobility characteristics through a separating gel.
3. A method according to claim 1 or 2, characterised in that the plurality of different probes (14) comprises a predetermined set of different probes (14) each chosen to be specific for a respective target nucleic acid sequence.
4. A method according to claim 3, characterised in that the set comprises probes (14) suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced enables quantitative determination of the copy number of the respective sequences in the sample.
5. A method according to claim 4, characterised in that the polymerase chain reaction is used to determine the quantity of each probe product produced.
6. A method according to any of the preceding claims, characterised in that the method is used to screen sequences of different genes or different

sequences within a gene, such as different exons in a eukaryotic gene.

7. A method according to any of the preceding claims, characterised in that the method is used to detect genetic alterations such as genetic deletions (reduction in sequence copy number) and genetic amplification (increase in sequence copy number).
8. A method according to any of the preceding claims, characterised in that the genetic material (10) is immobilised prior to hybridisation, such that hybridised flanking primers are likewise immobilised.
9. A method according to any of the preceding claims, characterised in that an excess of probes (14) is used.
10. A method according to any of the preceding claims, characterised in that probes (14) labelled for ready identification are used.
11. A method according to claim 10, characterised in that probes labelled with fluorescent labels are used.
12. A method according to any of claims 3 to 11, characterised in that more than one set of probes (14) is used, either simultaneously or sequentially.
13. A method according to claim 12, characterised in that the flanking primer pairs are the same for each set of probes (14).
14. A method according to claim 12, characterised in that the flanking primer pairs are different for each set of probes (14).
15. A method according to any of the preceding claims, characterised in that the method comprises means to obviate or mitigate hybridisation between primer binding sequences.

16. A method according to claim 15, characterised in that competing oligonucleotides are introduced to the sample preferably during the hybridisation stage to releasably bind to the primer binding sites flanking each probe whereby to mitigate primer binding site interactions.

17. A method according to any of the preceding claims, characterised in that unbound probes (14) and primers are thoroughly washed away from the bound probes (14) following hybridisation stage and prior to analysis.

18. A method according to any of claims 3 to 17, characterised in that the method is used to screen DNA, RNA and/or cDNA with appropriate probe sets.

19. A method according to claim 18, characterised in that the method is used to screen somatic and/or germline sequences.

20. A method according to claim 18 or 19, characterised in that the method is used to screen for polymorphic alterations.

21. A set of probes (14) substantially as described above in any of the preceding claims.

22. A kit for such a method described above in any of the preceding claims, which kit comprises a probe set (14) generally as defined above, amplification primers and means to enable amplification and analysis of amplification product(s).

23. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material substantially as hereinbefore described with reference to the accompanying examples and drawings.

24. A set of probes substantially as hereinbefore described with reference to the accompanying examples and drawings.

25. A kit for a method of screening for copy number of target nucleic acid sequences in a sample of genetic material substantially as hereinbefore described with reference to the accompanying examples and drawings.
26. Any novel subject matter or combination including novel subject matter disclosed herein, whether or not within the scope of or relating to the same invention as any of the preceding claims.

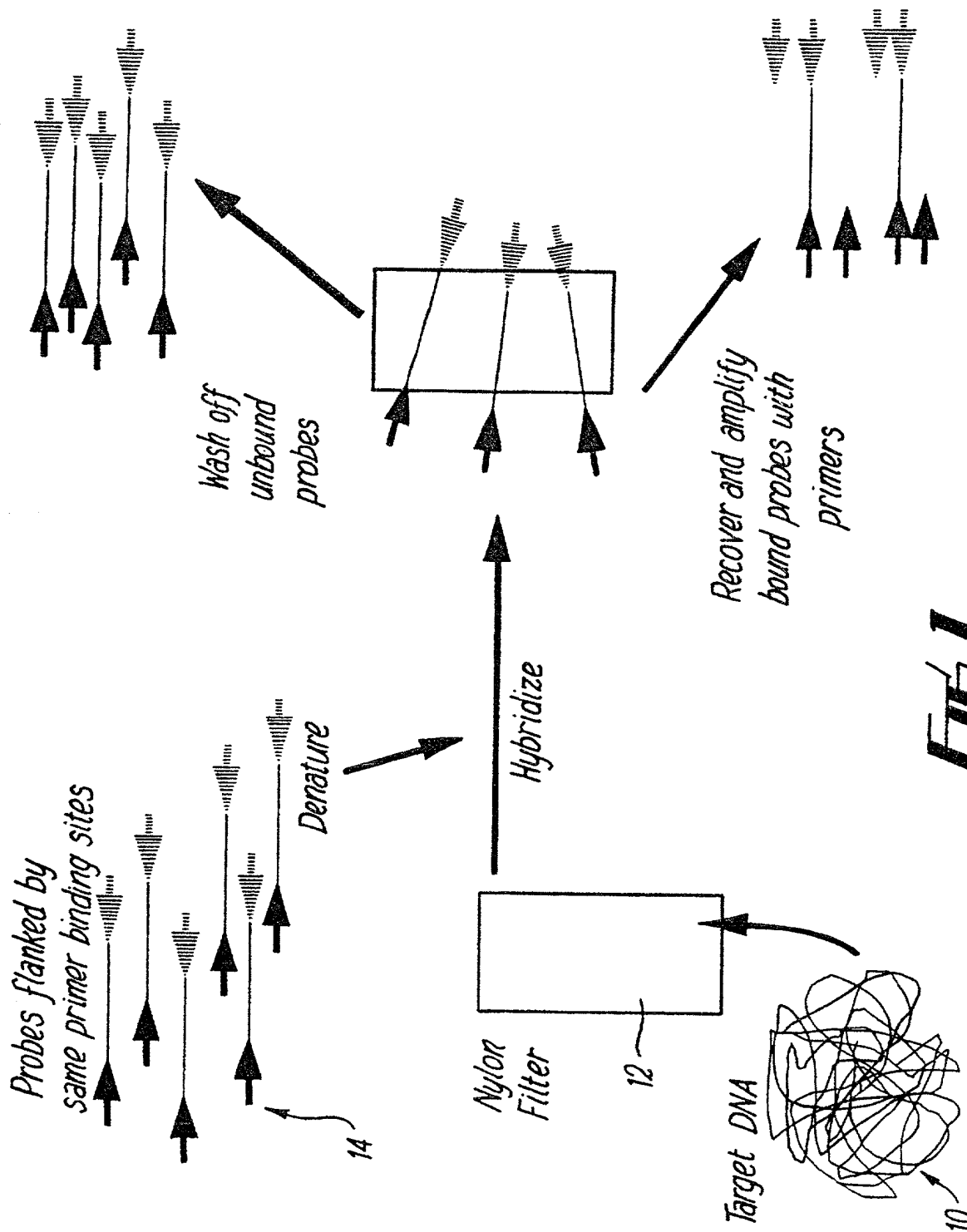
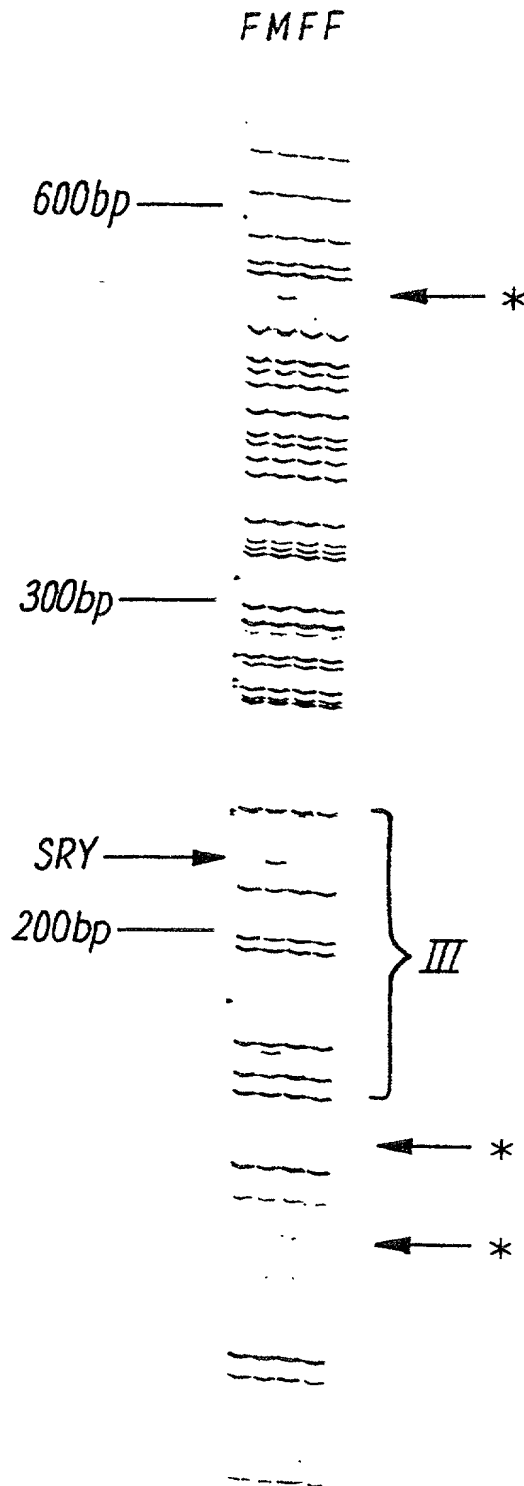
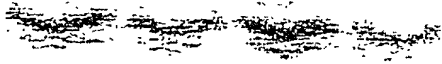


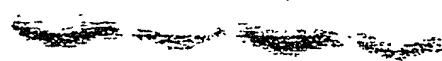
FIG. 1

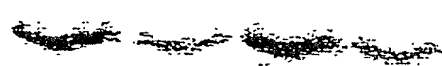
**FIG. 2**

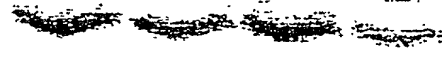
F M F F

22 → 

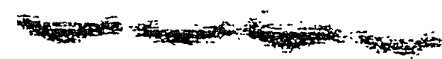
Y → 

X → 

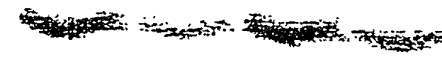
X → 

17 → 

15

22 → 

Y → 

X → 

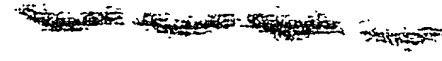
17 → 

Fig. 3

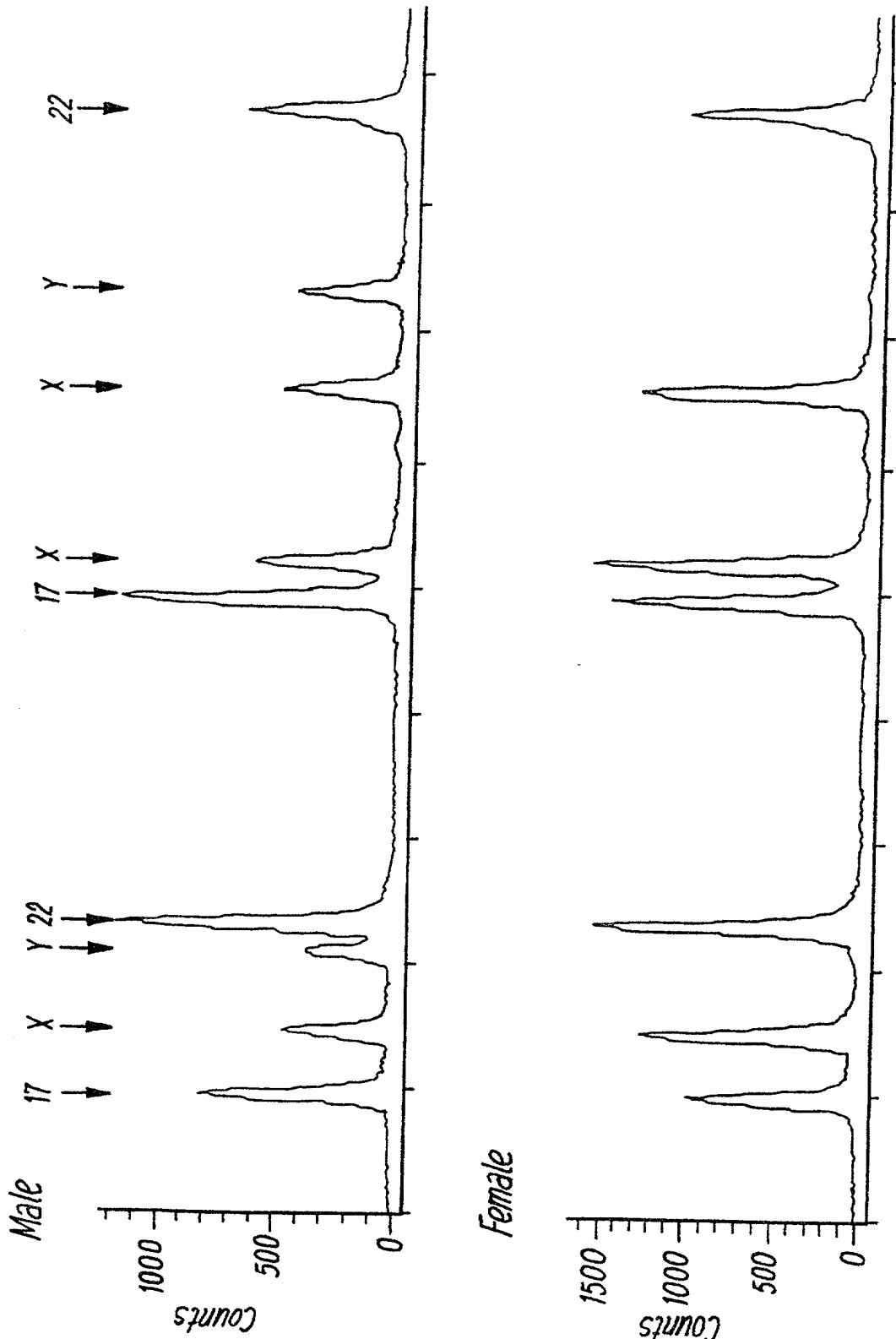
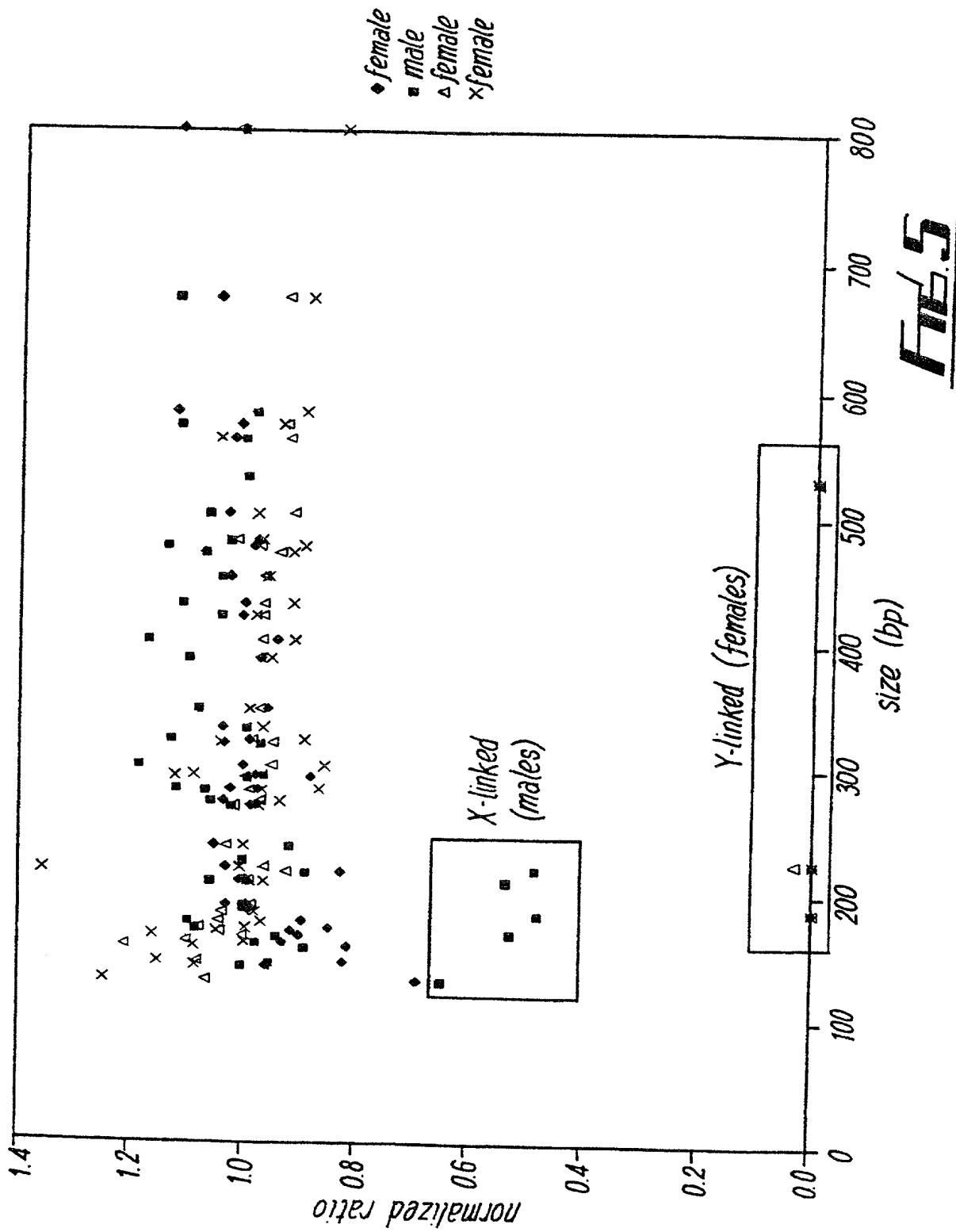


Fig. 4

1890	1891	1892	1893	1894	1895	1896	1897	1898	1899	1900	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914	1915	1916	1917	1918	1919	1920	1921	1922	1923	1924	1925	1926	1927	1928	1929	1930	1931	1932	1933	1934	1935	1936	1937	1938	1939	1940	1941	1942	1943	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298</
------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	--------



```

    primer PZA
      AGTAACGGCCGCGAGTGTGCTG      EcoRV
GGATCCACTAGTAACGGCCGCGAGTGTGCTGGAATTCTGCAGatatccATCACACTGGCGCGCTCGAGCA
+-----+-----+-----+-----+-----+-----+-----+-----+
CCTAGGTGATCATTGCCGGGGTCCACACGACCTTAAGACGTctatagTAGTGTGACCGCGCGAGCTCGT
      GTAGTGTGACCGCGCGGAGC
    primer PZB
  
```

Fig. 6

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)

 ATTORNEY'S DOCKET NUMBER
16-007

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

GENETIC SCREENING

the specification of which (check only one item below):

☐ is attached hereto.

☒ was filed as United States application

 Serial No. 09/914,811

 on August 30, 2001

and was amended

on _____ (if applicable).

☒ was filed as PCT international application

 Number PCT/GB00/00761

 on 3 March 2000 (03.03.00)

and was amended under PCT Article 19

on _____ (if applicable).

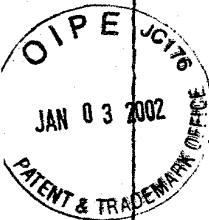
I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119;

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/GB00/00761	3 March 2000 (03.03.00)	5 March 1999 (05.03.99)
GREAT BRITAIN	9904991.3	05 March 1999 (05.03.99)	



09/914,811 03.03.00

DATE _____